Original Paper

Resveratrol Alleviates Inflammatory Responses and Oxidative Stress in Rat Kidney Ischemia-Reperfusion Injury and H₂O₂-Induced NRK-52E Cells via the Nrf2/TLR4/NF-κB Pathway

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Key Words

Resveratrol • Ischemia-reperfusion injury • Kidney • Inflammation • Oxidative stress

Abstract

Background: Ischemia-reperfusion injury (IRI) is one of the major causes of postoperative renal allograft dysfunction, which is mainly the result of proinflammatory reactions including inflammatory responses, oxidative stress, and metabolic disorders. Resveratrol (RSV) plays an important role in protecting various organs in IRI because it reduces oxidative stress, lessens the inflammatory response, and exerts anti-apoptotic effects. The aim of this study was to demonstrate the renoprotective effect of RSV in inhibiting inflammatory responses, reducing oxidative stress, and decreasing cell apoptosis *in vivo* and *in vitro*. **Methods:** RSV was administered before renal ischemia and H₂O₂ induction. Serum and kidneys were harvested 24 h after reperfusion and NRK-52E cells were collected 4 h after H₂O₂ stimulation. Serum creatinine and blood urea nitrogen were used to assess renal function. Hematoxylin and eosin staining was performed to assess histological injury. Quantitative real-time PCR and enzyme-linked immunosorbent assay were used to assess proinflammatory cytokine expression. Oxidative stress-related proteins, such as Nrf2 and TLR4, were evaluated by western blot. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay was used to detect apoptotic cells in tissues, and western blot was used to evaluate the expression of caspase-3, -8, and -9 in this study. **Results:** RSV inhibited inflammatory responses and improved renal function after renal IRI. Additionally, RSV decreased oxidative stress and reduced cell apoptosis by upregulating Nrf2 expression, downregulating the TLR4/NF-κB signaling pathway, and by decreasing caspase-3 activity and caspase cascades. **Conclusion:**

J. Li and L. Li contributed equally to this work.

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Our study demonstrated the mechanisms underlying RSV renoprotection. We found that RSV exerts its greatest effects by blocking inflammatory responses, lowering oxidative stress, and reducing apoptosis via the Nrf2/TLR4/NF- κ B pathway.

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Introduction

Ischemia-reperfusion injury (IRI) is one of the major causes of postoperative renal allograft dysfunction, leading to severe complications such as delayed graft function, acute kidney injury, and graft rejection [1, 2]. The pathophysiological mechanisms of IRI are primarily related to proinflammatory reactions, which include inflammatory responses, oxidative stress, and metabolic disorders [1, 3]. Resveratrol (RSV) is a stilbene polyphenolic compound found in grapes, berries, red wines, and peanut skins, and has been studied in many models such as coronary heart disease and diabetes due to its functions in reducing oxidative stress and inflammation [4-6]. In addition, many studies have shown that RSV can have protective effects on organs including the brain [7], heart [8], and kidney in animal IRI models because it reduces oxidative stress and exerts anti-inflammatory and anti-apoptotic effects [9, 10].

Oxidative stress enhances the concentrations of intracellular reactive oxygen species (ROS), in turn promoting proinflammatory cytokine release and cellular apoptosis. The nuclear factor erythroid 2-related factor 2 (Nrf2) is one of the regulators of antioxidant cell defense, as it acts as a protective transcription factor in IRI [11]. Kelch-like ECH-associated protein 1 (Keap1) binds Nrf2 in the cytoplasm, forming a complex that maintains Nrf2 at a low and steady level [12]. When IRI occurs, Keap1 undergoes conformational change and activates Nrf2, resulting in cellular protection against oxidative stress and enhancement of cell growth and survival [12, 13]. Hemeoxygenase-1 (HO-1) is one of the antioxidant response element (ARE)-dependent phase II detoxifying enzymes and antioxidants that are regulated by the redox-sensitive transcription factor Nrf2 [14-16]. Activation of Nrf2 induces HO-1 expression, suggesting that Nrf2 is essential for HO-1-mediated cytoprotection against IRI [17, 18].

Toll-like receptors (TLRs) are transmembrane proteins that play a key role in innate immunity [19]. Due to the reaction to oxidative stress in kidney IRI, the TLR4 signaling pathway is activated and expressed at a high level, causing a severe infiltration of inflammatory cells [20]. Nuclear factor (NF)- κ B is an important transcription factor regulating physiological processes, inflammatory responses, and apoptosis [21]. Normally, NF- κ B is sequestered in the cytoplasm by the I κ B family. Upon stimulation, IKKs phosphorylate I κ B family members, allowing NF- κ B to translocate to the nucleus. The IKK complex contains two I κ B kinases (IKK α and IKK β), which are involved in the inflammatory reaction [22]. In some pathological situations, NF- κ B is activated by stimuli from immune receptors such as TLRs and antigen receptors, which are upregulated by oxidative and genotoxic stress [21, 23]. Forkhead transcription factor O1 (FoxO1) plays a role not only in regulating metabolism but also in oxidative stress [24, 25]. The phosphorylation of FoxO by Akt blocks the FoxO DNA binding domain, leading to inhibition of FoxO1 transcriptional activity. Phosphoinositide-dependent kinase-1 (PI3K) activation of the serine/threonine protein kinase B (Akt) pathway plays a key role in inflammation. In animal models of IRI in the kidney and intestine, erythropoietin (a glycoprotein cytokine) has been shown to have significant protective effects against IRI. In a rat model of myocardial IRI, pretreatment with erythropoietin led to a significant decrease in the levels of proinflammatory cytokines [26].

As an antioxidant, RSV may protect against IRI through various mechanisms, including reducing ROS, activation of the TLR4 pathway, and caspase-3 activity. RSV has been shown to regulate the expression of TLR4, enhance the expression of Nrf2, and inhibit the activity of caspase-3 in cardiac and hepatic IRI [27-29]. Moreover, the protective effects of RSV have been demonstrated in many kidney diseases such as diabetic nephropathy, aldosterone-induced kidney injury, aging kidney, and even renal IRI [30], but the mechanism underlying

its renoprotective effect remains unclear. Furthermore, the mechanism of IRI and the protective functions of RSV are closely connected, suggesting that RSV may be effective in protecting against renal IRI. Therefore, we used a rat model for *in vivo* experiments and H_2O_2 -induced NRK-52E cells for *in vitro* experiments, with the aim of determining the underlying mechanisms through which RSV provides renoprotection in renal IRI. We report here, for the first time to our knowledge, that the renoprotective effect of RSV involves inhibiting inflammatory responses, reducing oxidative stress, and decreasing cell apoptosis via the Nrf2/TLR4/NF- κ B pathway.

Materials and Methods

Animals

Male Sprague-Dawley rats (200–250 g) were purchased from the Shanghai SLAC Lab Animal Co., Ltd. (Shanghai, China) and housed in temperature-controlled, SPF conditions with free access to food and water. Animals were fasted for 1 day prior to surgery. All animal procedures were performed in accordance with bioethics guidelines and were approved by the Bioethics Committee of Zhongshan Hospital, Fudan University, Shanghai, China.

Drugs

RSV was purchased from Sigma-Aldrich (St. Louis, MO) and diluted in 10% DMSO (Sigma-Aldrich) for storage at 4°C. Nrf2 siRNA used in this study was synthesized by Shanghai Gene Pharma Co., Ltd. (Shanghai, China).

Renal IRI model

Rats were randomly divided into four groups of five rats each: (1) sham group, (2) ischemia-reperfusion (IR) group, (3) RSV group, and (4) IR+RSV group. Renal IRI was induced by clamping the left renal artery for 60 min followed by right nephrectomy. Rats were anesthetized through intraperitoneal injections of pentobarbital sodium (40 mg/kg body weight). After a medial abdominal incision, the left renal artery was clamped for 60 min with a serrefine. Adequate restoration of blood flow after clamp removal was checked before abdominal closure. The right kidney was then removed. Sham-operated animals went through the same surgical procedure without clamping.

The IR group was treated with saline and received an intragastrical administration (1.5 mL) of 0.9% sterile NaCl for 30 min before renal clamping. The RSV group received intragastrical administration of RSV diluted in sterile saline to 0.23 μ g/kg body weight without renal clamping. The IR+RSV group received intragastrical administration (1.5 mL) of RSV diluted in sterile saline to 0.23 μ g/kg body weight for 30 min before renal clamping. After the operation, the rats were placed on a warming blanket for 12 h with food and water available. All animals were euthanized 24 h after surgery with an overdose of pentobarbital sodium, and their blood and kidneys harvested.

Cell culture and H_2O_2 -induced model

The rat tubular epithelial cell line NRK-52E was purchased from American Type Culture Collection (Manassas, VA) for use in this study. NRK-52E cells were cultured in Dulbecco's modified Eagle's medium (Gibco Technologies, Logan, UT) with 10% fetal bovine serum (Gibco) at 37°C in a 5% CO_2 atmosphere. NRK-52E cells were cultured in six-well plates and divided into seven groups (control group, RSV group, Nrf2 siRNA group, H_2O_2 group, H_2O_2 +Nrf2 siRNA group, H_2O_2 +RSV group, and H_2O_2 +RSV+Nrf2 siRNA group). H_2O_2 (Lingfeng, Shanghai, China) was administered at 500 μ mol/L. After H_2O_2 exposure for 4 h, cells were washed three times with PBS and cultured for further analysis. In the H_2O_2 +RSV and H_2O_2 +RSV+Nrf2 siRNA groups, RSV was added 1 h before H_2O_2 treatment, and the RSV concentration was 100 μ mol/mL [31].

Cell viability analysis

All groups of NRK-52E cells were washed twice with PBS, and then cultured in complete medium with 10% WST-8 (Beyotime, Shanghai, China) at 37°C for 1 h to form water-insoluble formazan. Complete medium containing 10% WST-8 was used as a control. A microplate reader (MDC, Hayward, CA) was used to measure the absorbance at 450 nm.

Assessment of renal function

Whole blood was centrifuged at 1, 600 g for 25 min at 4°C to obtain serum. An automated biochemical analyzer (Hitachi 7060, Tokyo, Japan) was used to measure the levels of serum creatinine (SCr) and blood urea nitrogen (BUN).

Histological assessment

Hematoxylin and eosin (HE) staining was performed to assess histological injury. Tissue sections (5 sections per kidney) were blindly labeled and randomly observed by two renal pathologists. Renal damage was graded based on the percentage of damaged tubules in the sample: 0 = no identifiable injury; 1 = mitosis and necrosis of individual cells; 2 = necrosis of all cells in adjacent proximal convoluted tubules, with survival of surrounding tubules; 3 = necrosis confined to the distal third of the proximal convoluted tubules, with a band of necrosis extending across the inner cortex; and 4 = necrosis affecting all 3 segments of the proximal convoluted tubule [32]. Injury included inflammatory cell infiltration, dilation of renal tubules, and interstitial edema. A score of 1 or 2 represents mild injury, and a score of 3 or 4 represents moderate or severe injury, respectively.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay

The TUNEL assay was used to detect apoptotic cells according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). Apoptotic cells were examined at 400× magnification over 20 fields of tubular areas.

Measurement of malondialdehyde (MDA) content, superoxide dismutase (SOD) activity, intracellular ROS activity, and glutathione (GSH) and oxidized glutathione (GSSG) analysis

MDA is a terminal product of lipid peroxidation and SOD is an important enzyme in oxidative stress. We used commercial kits (Beyotime, Nantong, China) to measure the concentration of MDA and a Total Superoxide Dismutase Assay Kit (Beyotime) to detect SOD activity in kidney tissues. ROS level can be used as an indicator of intracellular oxidative stress, so we used a Reactive Oxygen Species assay kit (Beyotime) to detect the intracellular level of ROS in all groups of NRK-52E cells. GSH and GSSG are important intracellular thiols. Alterations in the GSH/GSSG ratio were used to assess the exposure of cells to oxidative stress. The ROS level of cultured cells was measured using 2',7'-dichlorofluorescein diacetate, and the GSH/GSSG ratio was determined using a GSH/GSSG assay kit (Beyotime), according to the manufacturer's protocol.

Caspase-3 activity assays

We used caspase-3 colorimetric assay kits (KeyGEN, Nanjing, China) to detect relative caspase-3 activity in kidneys and NRK-52E cells. Optical density was measured at 405 nm with a microplate reader (MDC).

Western blot analysis

Renal tissue homogenates were prepared and the supernatant was maintained at 4°C. Protein from each sample (30 µg) was resolved in SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. NRK-52E cells were washed twice with PBS and then harvested. The membranes were incubated overnight at 4°C with primary antibodies including anti-cleaved caspase-3 (#9661S), anti-caspase-8 (#4790T), anti-caspase-9 (#9504S), anti-Nrf2 (#12721S), anti-Keap1 (#8047S), anti-HO-1 (#70081S), anti-NF-κB p65 (#8242S), anti-p-NF-κB p65 (#3033S), anti-IκB-α (#9242S), anti-p-IκB-α (#9246S), anti-IKKα (#2682S), anti-myeloid differentiation factor (MyD88, #4283S), anti-IKKα (#2682S), anti-TLR4 (#14358S) (Cell Signaling Technology, Danvers, MA, 1:1, 000) and anti-MMP-13 (#ab39012) (Abcam, Cambridge, UK, 1:3, 000), followed by a 1-h incubation with peroxidase-conjugated secondary antibodies (1:2, 000; Cell Signaling Technology) for 60 min in 5% non-fat milk at room

Table 1. Primers for quantitative real-time PCR

Gene	Primer sequence (5'–3')
IL-6	Upper: GCCCTTCAGGAACAGCTATGA
	Lower: TGTCAACAACATCAGTCCCAAGA
TNF-α	Upper: CCTTATCTACTCCAGGTTCTC
	Lower: GAGGCTGACTTTCTCCTGGTATG
IL-10	Upper: TGCAACAGCTCAGCGCA
	Lower: GTCACAGCTTTCGAGAGACTGGAA
IFN-γ	Upper: AAAGACAACCAGGCCATCAG
	Lower: CTTTTCGCTTCTTAGGCT
MMP-13	Upper: CATCCATCCCGAGACCTCAT
	Lower: GCATGACTCTCACAATGCGA
GAPDH	Upper: GGCACAGTCAAGGCTGAGAATG
	Lower: ATGGTGGTGAAGACGCCAGTA

temperature. Immunoreactive bands were visualized using ECL Western Blotting Substrate (Amersham Pharmacia, Sunnyvale, CA). As a loading control, the same membranes were simultaneously probed with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology, 5174S, 1:1,000). The signals were quantified by scanning densitometry using a Bio-Image Analysis System (Bio-Rad, Hercules, CA).

The results from each experimental group were expressed as relative integrated intensity compared with that of controls measured with the same batch. The appropriate negative control experiments were run for each target protein, as well as for β -actin and GAPDH, by replacing the primary antibody with normal immunoglobulin of the same species.

Quantitative real-time PCR

Total RNA was extracted from rat kidneys with TRIzol (Invitrogen, Carlsbad, CA) reagent according to the manufacturer's instructions. Total RNA (3–5 μ g) was transcribed into cDNA by Superscript II reverse transcriptase and random primer oligonucleotides (Invitrogen). The gene-specific primers for rat interleukin (IL)-6, tumor necrosis factor (TNF)- α , IL-10, and interferon (IFN)- γ are listed in Table 1.

Enzyme-linked immunosorbent assay (ELISA)

Serum levels of IL-6, IL-10, IFN- γ , and TNF- α were tested using ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. All experiments were performed with triplicate samples and repeated three times.

Statistical analyses

Data are presented as the mean $\pm$ standard error of the mean. Differences between any two groups were compared using two-tailed independent *t* tests, and means from three or more groups were compared by one-way analysis of variance. All statistical analyses were performed using SPSS 19.0 (IBM, Armonk, NY, USA), with *P* < 0.05 considered statistically significant.

Results

RSV alleviated renal dysfunction, ameliorated renal histologic damage, and decreased oxidative stress

Rats undergoing renal IRI showed tissue injuries in histological HE staining examination, which included inflammatory cell infiltration, dilation of renal tubules, and edema. We used a 0- to 4-point scoring system to evaluate tissue injury and found that injury scores were lower in the RSV-treated group than in the IR group (Fig. 1A). SCr and BUN concentrations were significantly increased in the IR group compared with the sham group. RSV

Fig. 1. Renoprotection by RSV in function and histology after renal IRI. (A) RSV ameliorated histological damage after IRI. Arrows indicate infiltrated lymphocytes. Representative images from one experiment out of three are shown and summarized results for HE staining scores and the number of infiltrated lymphocytes are shown as the mean $\pm$ standard error of the mean (SEM). (B) SCr and BUN concentrations were significantly higher in the IR group than in the sham group. Pretreatment with RSV inhibited renal dysfunction after renal IRI, and the RSV-only treatment group showed no significant differences from control animals. (C) RSV reduced MDA content and increased SOD activity in kidneys, indicating a reduction in oxidative stress. (B, C) Results are the mean $\pm$ SEM from three independent experiments (*n* = 5 mice per group). ***P* < 0.01; ****P* < 0.001.

pretreatment reduced the increase in SCr and BUN concentrations after IRI (Fig. 1B). Additionally, results from the RSV group showed that the HE staining score was 0 and the filtration of lymphocytes showed that lymphocytes increased after IRI (Fig. 1A). Concentrations of SCr and BUN in the RSV group were nearly equal to the sham group (Fig. 1B).

Reoxygenation is a major factor in IRI and always follows IRI causing tissue oxidative stress. We determined MDA content and SOD activity to investigate the influence of RSV on oxidative stress. We found that following renal IRI, MDA content was substantially increased and SOD activity was reduced, indicating that IRI increased oxidative stress. However, compared with the IR group, RSV pretreatment reduced the upregulation of MDA content and further downregulated SOD activity, indicating that RSV can reduce oxidative stress following IRI. Furthermore, there was no significant difference in MDA content between the RSV group and the sham group, but SOD activity showed a slight increase in the RSV group (Fig. 1C).

RSV decreased oxidative stress and inflammatory reactions, downregulated TLR4, MyD88, and NF-κB signaling, and inhibited subsequent proinflammatory responses

The mRNA expression of proinflammatory cytokines IL-6, IFN-γ, and TNF-α increased after IRI and was reduced by RSV treatment, similarly to MMP-13. IL-10, a classic anti-inflammatory factor, was increased in the IR+RSV group, suggesting the anti-inflammatory effect of RSV (Fig. 2A). The ELISA experiments showed similar results (Fig. 2B).

After IRI, the expression of TLR4 increased, as did MyD88, a general adaptor protein for TLR4. NF-κB is an essential downstream effector of TLR4/MyD88 signaling, which can enhance proinflammatory responses. The NF-κB inhibitor IκB-α and its regulator IKKα were

Fig. 2. Alleviation of proinflammatory responses by RSV. (A, B) RSV reduced the mRNA and protein expression of proinflammatory cytokines induced by IRI. (C) IRI activated the TLR4/NF-κB signaling pathway; however, RSV treatment decreased TLR4 and MyD88 expression and suppressed p-NF-κB and p-IκB-α. MMP-13 increased significantly in the IR group, and slightly decreased in the IR+RSV group. (D) Nrf2 and HO-1 were increased in the IRI and IR+RSV groups, but Keap1 decreased in the IR+RSV group. (A, B) Results are the mean ± SEM from three independent experiments (n = 5 mice per group). (C, D) Results are representative of three independent experiments and summarized results are shown as the mean ± SEM. *P<0.05; **P<0.01; ***P<0.001.

also evaluated in our study (Fig. 2C). Based on our results, phosphorylation of NF- κ B and I κ B differed significantly between the IR and sham groups. The expression of related inflammatory proteins such as TLR4, MyD88, IKK α , p-I κ B- α , and p-NF- κ B decreased significantly after RSV treatment, with the exception of NF- κ B inhibitor I κ B- α , which increased significantly (Fig. 2C).

Nrf2 is a key antioxidant transcription factor in eukaryotes, playing an important role in adaptive response. Keap1 has been shown to interact with Nrf2, expressed as a Keap1-Nrf2 complex in defensive responses against oxidative stress. HO-1 is one of the ARE-dependent phase II detoxifying enzymes and antioxidants that are regulated by Nrf2. Activation of Nrf2 induced HO-1 expression, suggesting that Nrf2 is essential for HO-1-mediated cytoprotection against IRI (Fig. 2D). We measured protein levels in the different groups to identify the degree of oxidative stress. Results showed that IRI upregulates the expression of Nrf2 and Keap1, and RSV treatment promotes the expression of Nrf2, suggesting that IRI is likely related to the adaptive response, activated by RSV (Fig. 2D).

RSV downregulated the activity of caspase-3 and decreased apoptosis following IRI

Mitochondrial dysfunction occurs after IRI and causes cell apoptosis. To evaluate the degree of dysfunction and apoptosis, we used TUNEL and western blot assays in our study. TUNEL staining showed that cell apoptosis increased after IRI, with most apoptotic cells located in tubular areas. RSV treatment significantly decreased the level of cell apoptosis (Fig. 3A), and also resulted in significant inhibition of caspase-3 activity (Fig. 3B) and decreased expression of cleaved caspase-3 (Fig. 3C) after kidney IRI. Caspase-8 and caspase-9 were both upregulated in the IR group, strongly indicating the involvement of mitochondrial stress in IRI. However, in the RSV treatment group, the expression of caspase-8 and caspase-9 was downregulated (Fig. 3C), suggesting that RSV reduces apoptosis through inhibition of mitochondrial stress.

RSV pretreatment increased cell viability, decreased oxidative stress, and downregulated TLR4/NF- κ B signaling after H₂O₂ stimulation of NRK-52E cells

NRK-52E cells treated with H₂O₂ showed a significant reduction in cell viability, which was prevented by RSV pretreatment (Fig. 4A). To evaluate the level of oxidative stress in the cells, we examined the intracellular ROS level. Based on our results, H₂O₂ stimulation greatly

Fig. 3. Effect of RSV in reducing mitochondrial stress and apoptosis. (A) The number of apoptotic cells in the IR group increased significantly according to the TUNEL assay, but was greatly reduced by RSV treatment. (B) IRI upregulated the activity of caspase-3, and RSV inhibited it. Results are the mean $\pm$ SEM from three independent experiments (n = 5 mice per group). (C) Upregulated expression of caspase-8, caspase-9, and cleaved caspase-3 in IRI was suppressed by RSV treatment, suggesting an anti-apoptotic function. (A, C) Results are representative of three independent experiments and summarized results are shown as the mean $\pm$ SEM. *P<0.05; **P<0.01; ***P<0.001.

Fig. 4. Alleviation by RSV of the proinflammatory response in an *in vitro* model. NRK-52E cells were divided into seven groups (control group, RSV group, Nrf2 siRNA group, H_2O_2 group, H_2O_2 +Nrf2 siRNA group, H_2O_2 +RSV group, and H_2O_2 +RSV+Nrf2 siRNA group). H_2O_2 was administered for 4 h at 500 μ mol/L and RSV was administered 1 h before H_2O_2 treatment at 100 μ mol/mL. Cell viability (A), intracellular ROS level (B), and GSH/GSSG ratio (C) were measured 4 h after cell culture. (D) Levels of TLR4, MyD88, IKK α , NF- κ B, p-NF- κ B, I κ B- α , p-I κ B- α , MMP-13, and GAPDH from cultured cells were determined by Western blot analysis. (E) Levels of Nrf2, Keap1, HO-1, and GAPDH from cultured cells were determined by Western blot analysis. (A–C) Data are shown as the mean $\pm$ SEM from three independent experiments ($n = 5$). (D, E) Results are representative of three independent experiments and summarized results are shown as the mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

increased ROS in the IR group, while RSV reduced ROS levels after H_2O_2 stimulation (Fig. 4B), suggesting that

resistance to H_2O_2 injury was improved by RSV. The GSH/GSSG ratio also reflects oxidative stress in cells. The GSH/GSSG ratio was downregulated by H_2O_2 treatment, while RSV pretreatment upregulated the GSH/GSSG ratio in NRK-52E cells (Fig. 4C). However, silencing of Nrf2 by siRNA in NRK-52E cells reversed the beneficial effects of RSV (Fig. 4A–C).

In the rat IRI model, we examined the TLR4/NF- κ B signaling pathway to assess proinflammatory responses. In the *in vitro* model, we used western blot assays to evaluate protein expression after H_2O_2 stimulation and RSV pretreatment. According to our results, TLR4/NF- κ B signaling was activated after H_2O_2 stimulation, and phosphorylation of NF- κ B and I κ B- α also increased significantly (Fig. 4D). RSV displayed its anti-inflammatory function by reducing the expression of the proinflammatory cytokines TLR4, MyD88, IKK α , p-I κ B, and p-NF- κ B, and the NF- κ B inhibitor I κ B- α also increased after H_2O_2 stimulation with RSV pretreatment (Fig. 4D), suggesting RSV inhibited proinflammatory responses after H_2O_2 stimulation. To further elucidate the role of Nrf2 in the TLR4/NF- κ B signaling pathway, Nrf2 was silenced in NRK-52E cells. As shown in Fig. 4D, the expression of TLR4, MyD88, IKK α , NF- κ B, p-NF- κ B, p-I κ B- α , and MMP-13 was upregulated, while NF- κ B inhibitor I κ B- α was decreased with Nrf2 silencing after H_2O_2 stimulation. Furthermore, silencing of Nrf2 reversed the beneficial effects of RSV (Fig. 4D).

The Keap1-Nrf2 complex, an essential component in defending against oxidative stress reactions, and HO-1 were increased after H_2O_2 treatment. However, RSV pretreatment

upregulated the Nrf2 level, indicative of the anti-oxidative function of RSV, the effects of which can also be suppressed by silencing Nrf2 (Fig. 4E).

RSV pretreatment inhibited caspase-3 activation and cell apoptosis after H₂O₂ stimulation in NRK-52E cells

The caspase cascade induces cell apoptosis after oxidative stress, and caspase-3, a downstream effector in the cascade, can directly mediate apoptosis after caspase signaling activation. We found that caspase-3 activity increased markedly after H₂O₂ stimulation and that RSV treatment reduced this activity (Fig. 5A), suggesting the ability of RSV to reduce cell apoptosis. The expression of caspase-8, caspase-9, and cleaved caspase-3 in NRK-52E cells also increased significantly after H₂O₂ stimulation (Fig. 5B), indicating that H₂O₂ stimulation caused severe cell apoptosis. Silencing of Nrf2 accelerated cell apoptosis and also reversed the beneficial effects of RSV (Fig. 5B). Based on our results, we conclude that RSV pretreatment decreases cell apoptosis (Fig. 5B), suggesting it has an anti-apoptotic function.

Discussion

RSV, a natural polyphenol, has potentially beneficial properties including reducing oxidative stress and decreasing inflammatory responses [9, 33, 34]. To the best of our knowledge, our study is the first to show the involvement of the Nrf2/TLR4 signaling pathway in RSV-mediated protection against renal IRI using *in vivo* and *in vitro* models. In renal IRI, RSV treatment alleviated kidney dysfunction via anti-inflammatory and anti-oxidative effects. In addition, RSV reduced cell apoptosis in the rat renal IRI model and in H₂O₂-induced NRK-52E cells, suggesting its role in protecting renal tubular epithelial cells. Furthermore, inhibition of Nrf2, TLR4, and the NF-κB pathway by RSV also implicated these proteins in the mechanism of RSV renoprotection.

Kidney transplantation is an important method for the treatment of end-stage kidney disease. Although surgery can save patients' lives, severe postoperative complications may occur. IRI is a major complication in kidney transplantation, and has been shown to be responsible for delayed graft function, allograft losses, and renal failure [35]. The pathophysiological mechanisms of IRI include oxidative stress reactions, inflammatory

Fig. 5. Anti-apoptotic effect of RSV in an in vitro model. NRK-52E cells were divided into seven groups (control group, RSV group, Nrf2 siRNA group, H₂O₂ group, H₂O₂+Nrf2 siRNA group, H₂O₂+RSV group, and H₂O₂+RSV+Nrf2 siRNA group). H₂O₂ was administered for 4 h at 500 μmol/L and RSV was administered 1 h before H₂O₂ treatment at 100 μmol/mL. (A) Relative caspase-3 activity in NRK-52E cells was measured using caspase-3 colorimetric assay kits. Data are shown as the mean ± SEM from three independent experiments (n = 5). (B) Levels of caspase-8, caspase-9, cleaved caspase-3, and GAPDH from cultured cells were determined by Western blot analysis after H₂O₂ stimulation. Results are representative of three independent experiments and summarized results are shown as the mean ± SEM. *P<0.05; **P<0.01; ***P<0.001.

responses, and cell apoptosis [2, 36]. Therefore, inhibiting the inflammatory responses and reducing cell apoptosis have become an important focus in treating renal IRI [36].

Oxidative stress is due to an imbalance between ROS production and antioxidant defenses. Exogenous antioxidants or the modulation of antioxidant enzymes can be expected to reduce oxidative stress. During IRI, the damaged tissue produces excessive amounts of ROS, causing oxidative stress that results in mitochondrial oxidative phosphorylation, ATP depletion, increases in intracellular calcium, and activates membrane phospholipid proteases [37]. Nrf2 and Keap1 are two major proteins in the immune reaction that act as a complex in the cytoplasm to control intracellular oxidative stress [13, 38]. Tan *et al.* found that upregulating Keap1 in hypomorphs protected mice against IRI, and was associated with a reduction in inflammatory gene expression and upregulation of Nrf2 target genes [39]. As a natural antioxidant, previous studies have shown that RSV can directly scavenge ROS, and exogenously administered RSV modulates the expression and activity of antioxidant enzymes such as SOD, glutathione peroxidase, and catalase through transcriptional regulation via Nrf2, activator protein 1, FoxO, and SP-1 or through enzymatic modification [30]. Our data are consistent with these studies, demonstrating that RSV treatment dramatically upregulates Nrf2 expression while downregulating Keap1 expression both *in vivo* and *in vitro*, and silencing of Nrf2 significantly reversed the beneficial effect of RSV. HO-1 is a stress-response protein that is highly inducible in response to pathological stimulation and serves as a protective mechanism against oxidative injury [40]. According to our results, expression of HO-1 was inhibited by silencing Nrf2 expression after RSV treatment, suggesting that Nrf2 may be a major target of RSV in relieving oxidative stress. In a mouse liver IRI model, the activation of Nrf2 was shown to regulate the TLR4 pathway and inhibit innate immunity [24, 41]. Taken together, these studies and our results implied a connection between oxidative stress and inflammatory responses, indicating that RSV may reduce oxidative stress injuries first and regulate innate immunity in a further step.

The TLR4 signaling pathway, which is an important inflammatory cascade in IRI and can be regulated by the activation of Nrf2, has been shown to have essential functions in innate and adaptive immune systems [42]. TLR4 is a type of immune receptor expressed on cell surfaces that can be activated by IRI, triggering the TLR4 signaling pathway [43]. The TLR4-activating signal is transmitted through the adaptor protein MyD88, which is associated with activated TLRs and enhances further signaling cascades that activate the inflammation-related protein NF- κ B [44, 45]. In IRI, NF- κ B is activated by phosphorylation of I κ B, leading in turn to activation of the IKK complex, which regulates proinflammatory responses [22, 23, 46]. In our study, we found that the upregulation of TLR4 and MyD88 induced by IRI or H₂O₂ stimulation was downregulated by RSV treatment. Furthermore, RSV treatment in the rat IRI model also resulted in significantly reduced expression of p-NF- κ B and p-I κ B, while increasing the expression of I κ B, compared with the IR group. With regard to H₂O₂-induced NRK-52E cells, p-NF- κ B and p-I κ B were significantly upregulated after H₂O₂ stimulation and downregulated by RSV pretreatment. In contrast, the expression of I κ B increased in the H₂O₂ group and was diminished by RSV. Consequently, our results indicate that RSV inhibits the TLR4/NF- κ B signaling pathway in the rat IRI model and in H₂O₂-induced cells, which may be the principle mechanism underlying the inflammatory responses produced in IRI by oxidative stress.

FoxO1 plays a role not only in regulating metabolism but also in oxidative stress [24]. Phosphorylation of FoxO by Akt blocks the FoxO DNA binding domain, leading to inhibition of FoxO1 transcriptional activity. Chen *et al.* reported that RSV regulates the expression of FoxO target genes and might regulate cell survival and/or apoptosis through global modulation of gene expression via deacetylation of FoxO transcription factor [47]. Kawabata *et al.* found that RSV could reduce the migration of osteoblasts via suppression of Akt [48]. While our study focused on the effects of RSV on the Nrf2/TLR4/NF- κ B pathway, the Akt/FoxO axis cannot be excluded from contributing to the protective effects of RSV in IRI, although elucidation of the precise mechanisms requires further study.

IRI leads to both necrosis and apoptosis of renal tubular cells, resulting in the release of damage-associated molecular pattern molecules and propagation of inflammation [49]. Cell apoptosis increases where it occurs pathologically, leading to tissue injuries and loss of function [50]. The results of TUNEL assays showed that RSV treatment reduces the number of apoptotic cells increased by IRI, indicating that RSV has an anti-apoptotic effect. Due to oxidative stress reactions in IRI, the increasing intracellular ROS level stimulates mitochondria-mediated apoptosis. Caspase-8 and caspase-9 are two important indicators of mitochondria-mediated apoptosis [51, 52], and our results showed that RSV downregulated both factors *in vivo* and *in vitro*, indicating that RSV reduced cell apoptosis by downregulating the caspase cascade. The caspase cascade also includes another effector, caspase-3, which can be activated in IRI and directly mediates cell apoptosis. We found that RSV inhibited caspase-3 activity and downregulated its expression in both the rat IRI model and in H₂O₂-induced NRK-52E cells.

In conclusion, our study is the first to show the mechanism of RSV renoprotection in both *in vivo* and *in vitro* models. We suggest that RSV exerts its most significant effects on inflammatory responses, oxidative stress, and apoptosis via the Nrf2/TLR4/NF-κB pathway.

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Disclosure Statement

No conflict of interests exists.

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